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Effects of gamma irradiation and comparison of different extraction methods on sesquiterpene lactone yields from the medicinal plant *Thapsia garganica* L. (Apiaceae)

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Abstract

Ethnopharmacological relevance

Thapsia garganica L. roots are used in Algerian traditional medicine for a number of ailments. It is used in a poultice as an antitussive treatment of acute bronchitis and pneumonia, in preparations with milk or oil taken orally to treat common lung diseases, and with the direct application of root sections for the soothing of dental pains.

Aim of the study

The objective of this study was to evaluate the combined effect of microwave assisted extraction and gamma irradiation on sesquiterpene lactones in *T. garganica* extracts

Materials and methods

To evaluate the combined effect of microwave assisted extraction and gamma irradiation on the highly bioactive compounds found in extracts of Algerian *T. garganica*, samples from different locations in Algeria were prepared by extraction from dried leaf and root samples of dried plant material, using different extraction methods. Quantification of the compounds of interest was done using an HPLC. The antioxidant activity extracts was determined using the two free radical scavenging assays: the 2,2-diphenyl-picryl-hydrazyl (DPPH) and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS).

Results

It was found that location and extraction method had significant impact on the phytochemical composition of extracts. Gamma irradiation was found to have no effect on the phytochemical composition of the plant extracts or on their antioxidant properties.

Conclusion

The study has shown that microwave assisted extraction is an effective method for investigating chemical compounds in *T. garganica* and the results support the notion that gamma irradiation for sterilization do not alter the chemical composition.

The authors wish to clarify that we cannot recommend the usage of any parts of T. garganica, in any form, for any remedy due to its very high toxicity.

Keywords: *Thapsia garganica*; gamma irradiation; microwave assisted extraction; thapsigargin; antioxidant

1. Introduction

1.1. Traditional use in Algeria

Thapsia garganica L. (Apiaceae) is a medicinal plant commonly found in Algeria, along the coast, in the plains, in the Saharan Atlas Mountains and in the north of the Saharan desert (Hammiche et al., 2013). It is commonly referred to as: Toufelt in Berber; adhriss by the Kabyle people in the North; thapsie, bounafaa or bou-nafit «that of efficacy» in Arabic; faux fenouil (false fennel) and Thapsia du mont Gargan in French(Hammiche et al., 2013). In English, it is known as the deadly carrot. All parts of the plant are known to be toxic and irritant to the skin, causing blisters, erythema and itching, and the resin of the roots has been found to be particularly toxic (Andersen et al., 2015b). Due to this toxicity *T. garganica* is not allowed in any official pharmaceutical preparation, and we cannot recommend the usage of *T. garganica* roots or fruits, in any form, for any remedy due to its very high toxicity. *T. garganica* roots are however used in Algerian traditional medicine for a number of ailments. In Kabylia, the Kabyle people use the root to make a "depurative cure" at the onset of spring (Hammiche et al., 2013). They also use the roots to make a poultice, which is applied to the chest as an antitussive treatment of acute bronchitis and pneumonia. Great care is taken in the preparation and its use is limited; in fact, it is a treatment of last resort when bad weather prevents travel (Hammiche, 2015). If the medical condition is less severe, the oil in which a fresh root is cooked is either rubbed on the chest for its "purgative" properties or ingested in small quantities (Hammiche, 2015). Other traditional uses in Algeria include a preparation with milk or oil taken orally to treat common lung diseases, and the soothing of dental pains with the direct application of root sections (Hammiche et al., 2013).

The toxicity of *T. garganica* originates from the presence of thapsigargin (Fig. 1) and other sesquiterpene lactones (Andersen et al., 2015a; Andersen et al., 2017; Drew et al., 2009; Simonsen et al., 2013). Thapsigargin makes up 0.2-1.2% of the dry weight of the plant's roots (Andersen et al., 2015b). The pharmacological activity of thapsigargin is due to its inhibition of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in mammalian cells, which leads to cell apoptosis (Simonsen et al., 2013).

1.2. Antioxidant activity

Both the food and pharmaceutical industries have shown a continuing interest in finding naturally occurring antioxidants for use in the preservation of foods or medicinal products, in order to replace synthetic antioxidants, which are being restricted due to their carcinogenicity

and harmful effects on the environment (Prakash et al., 2015). Essential oils from aromatic and medicinal plants, in particular, have been of special interest due to their strong antioxidant activity and antimicrobial constituents in their tissues (Di Venere et al., 2016; Golubović et al., 2014). It has previously been seen that certain Algerian medicinal plants, including *T. garganica*, contain strong radical scavengers and can therefore be useful as sources of natural antioxidants for both medicinal and commercial use (Djeridane et al., 2006). However, as many of these plants contain toxic compounds, toxicity issues need to be addressed to ensure the antioxidants are safe to use.

1.3. Irradiation of medicinal herbs

Medicinal plants are widely used in Algerian folk medicine, especially by the elderly and rural communities with limited access to doctors. However, the plants are subject to deterioration from chemical and microbial processes that occur before reaching the end-user during harvesting, processing, distribution and storage. These processes can alter their efficacy and in some cases their safety, so there is a demand for methods of decontamination and preservation in order to improve consumer safety and therapeutic efficacy. Food irradiation is commonly used to sterilise and to reduce food losses due to spoilage, and it has replaced once commonly used chemical fumigants, like ethylene oxide, and other chemical preservatives that have been reported to be hazardous to human health (Seo et al., 2007). The use of gamma irradiation on food products is approved by the Food and Agriculture Organisation (FAO), the International Atomic Energy Agency (IAEA) and the World Health Organisation (WHO)(Joint, 2009). It has been shown to be a safe, environmentally friendly and energy efficient method to sterilise plant products. It is also a well-established industrial process for the sterilisation of medicinal plants in a number of facilities worldwide and in general do not affect the chemical composition of the leaves and roots (Garg and Gupta, 2016; Seo et al., 2007).

1.4. Extraction methods

Traditional extraction methods of medicinal plants include decoction or maceration in an organic solvent. These methods however, are highly energy dependent and time consuming. Microwave assisted extraction (MAE) has been found to be a reliable alternative as it requires a lower energy input to result in the same or even higher extraction yields, reduces the use organic solvents, shortens extraction times and improves the reproducibility of results. This extraction method has been used for the analysis of bioactive compounds in a number of medicinal plants (Akloul et al., 2014; Benkaci-Ali et al., 2006; Kennouche et al., 2015).

However, care should be taken to choose suitable conditions to avoid the thermal degradation of the analytes of interest. Sample preparation and extraction methods are important to consider when studying medicinal plants, as the methods chosen depend on the target compounds and can affect the phytochemical composition of the final extracts.

Here we investigate the chemical composition of extracts of *T. garganica* from different regions in Algeria. We evaluate the combined effects of microwave assisted extraction and gamma irradiation on the extraction yield of the bioactive compounds as well as on the antioxidant activity of the extract.

2. Materials and Methods

2.1. Plant material

Thapsia garganica L. (Apiaceae) roots and leaves were collected between March and April in 2014 and 2015 during flowering, from two locations in Algeria: Médea (Aïn Boucif) (GPS coordinates N35° 53' 28"/E3° 9' 31") and Béjaia (Kherrata) (GPS coordinates N36° 29' 34"/E5° 16' 39"). At each site 50 individuals were sampled. For each individual representative leaf material was taken across the entire plant and roots were dug out. Herbarium vouchers were made for one individual per site. The herbarium vouchers are deposited at the Natural History Museum of Denmark, Herbarium C (C10011584, C10011585; leg. Abir Mohamed Mohamed Ibrahim). The local name, the used plant parts, methods of preparation and administration, and medicinal uses were collected from local inhabitants. Samples were identified Dr. Abdelkrim of the Botanical department at the National School Agronomic, Algiers, Algeria, air-dried and stored at room temperature in the laboratory of chemistry. The collections were made according to Algerian regulations.

2.2. DNA extraction, amplification and sequencing

The taxonomy and species concept of *Thapsia* is not resolved (Weitzel et al., 2014), thus to confirm the identity of the collected samples, total genomic DNA from one sample per site (herbarium accession numbers: C10011584, C10011585) was extracted from 15mg of dried leaf fragments, using the QiagenDNeasy Kit (Qiagen, Copenhagen, Denmark) following the manufacturer's protocol. The nuclear ribosomal internal transcribed spacer (nrITS) region was sequenced as described previously (Weitzel et al., 2014), using primers ITS4 and ITS5. Sequences were edited and assembled using CLC Main Workbench 7 software. BLAST analysis confirmed material from both sites to be *T. garganica* (99% match) as previously

identified (Weitzel et al., 2014). The new sequences generated are deposited in GenBank, with the following accession numbers: (submitted to GenBank).

2.3. Irradiation

200g of dried root and leaf samples were subjected to the following doses (D) of gamma radiation (values in KGy): D_1:0.1, D_2:0.3, D_3:0.7, D_4:1, D_5:3, D_6:7 and D_7:10; at room temperature in the Centre of Nuclear Research Algiers, Algeria (Centre de Recherche Nucléaire d'Alger, CRNA) with a ^{60}Co source. Non-irradiated (D_0:0kGy) samples were used as negative controls. The irradiated samples were kept in the dark and at room temperature (ca 22°C) until analysis.

2.4. Extraction and fractionation of plant material of T. garganica for HPLC quantification

The individual samples of leaves and roots collected at each site were pooled to make a representative sample of leaves and roots for each area.

Simple extraction (SE): 1.5 mL of organic solvent (1:1 mixture of 80% MeOH in water and 80% Acetone in water) was added to 50 mg of homogenised dried and ground plant material using liquid nitrogen (-196°C) to preserve the samples, then vortexed thoroughly and agitated overnight in a thermomixer (Eppendorf® Thermomixer Compact) at 850 rpm at 25 °C. Samples were then centrifuged for 10 min at 10,000 rpm. 1 mL of the supernatant was evaporated to total dryness in a vacuum concentrator (Scan Speed Maxi Vac Evaporator). 250 µL of 80% methanol was then added to re-suspend the extract for HPLC analysis.

Classical maceration (CM): 40 g samples of dried and ground roots and leaves (irradiated and untreated) of *T. garganica* were submerged in 100 mL of methanol at 40 °C for 10 h under magnetic stirring. After filtration, the methanol extracts were concentrated under reduced pressure to obtain crude extracts and then lyophilized to eliminate all trace of solvent and stored at 4 °C. Before HPLC analysis, 1 mg of each sample was re-suspended in 1 mL 100% methanol.

Microwave assisted extraction (MAE): 40 g of each dried sample (irradiated and untreated) were ground to powder. Samples were then extracted in 100 mL methanol in a microwave device as previously described (Akloul et al., 2014) for 30 min. The resultant mixture was filtered under vacuum and the filtrate was evaporated to near dryness. The samples were then completely lyophilized and stored at 4 °C. 1 mg of each dry extract was re-suspended in 1 mL 100% methanol before HPLC analysis.

2.5. Chemical Standards

Standards extracted from the fruits of *T. garganica* were used as references for the identification and quantification of thapsigargin (Tg), nortrilobolide (Nb) and thapsigargicin (Tc) (donated by Søren Brøgger Christensen, University of Copenhagen, Denmark). Standard solutions were prepared in triplicate, diluted in 80% methanol (standard dilutions: 5, 25, 50, 200, 400, 500, 600, 800, 1000 µg/mL).

2.6. HPLC analysis

All samples were filtered in centrifugal filters (Ultrafree® MC GV, 0.22 µm Durapore® PVDF) just before injection into the HPLC. HPLC analysis was performed on an Analytical HPLC-UV Shimadzu Prominence (column oven 30 °C, autosampler 15 °C) and performed on a Kinetix EVO C18 100A column (5 µm, 50 mm× 3 mm; Phenomenex). Acetonitrile (solution A) and milliQ water (solution B) were used as the mobile phase with a flow rate of 0.5 mL/min. The gradient program was as follows: 50% A (0–1 min, linear gradient), 100% A (6–9 min, linear gradient), 5% A (14-16 min, linear gradient), 50% A (17-23 min, linear gradient) the flow rate was fixed at 0.5 mL/min. Eluting compounds were detected with UV at 230 nm. Each sample was prepared in triplicate and 10 µl was injected into the HPLC. Calibration curves were generated based on triplicate analysis. To obtain a standard curve for quantification, the calibration graphs were linear in the concentration range 5-1000 µg/mL. The calibration curves for each standard had a correlation coefficient of 0.999.

2.7. Antioxidant activity

The antioxidant potential of *T. garganica* root and leaf extracts was determined using the two most widely used free radical scavenging assays: the 2,2-diphenyl-picryl-hydrazyl (DPPH) and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS). All experiments were performed in triplicate for the different concentrations of each plant extract.

DPPH assay: The free radical scavenging capability of each extract solution was measured from the bleaching of a purple solution of DPPH as described previously (Şahin et al., 2004). 1 mL of methanol solution of 60 µM DPPH was mixed with 26 µL of each of the methanolic extracts of the roots and leaves at different concentrations, 100-1000 mg/mL and 25-200 mg/mL respectively. The reaction mixture was carried out in capped glass test tubes. After 30min of incubation at room temperature, the absorbance was measured at 517 nm using an

optizen Mecasys spectrophotometer. The inhibition percentage of DPPH free radicals (I%) was calculated as follows:

$$I\% = (A_0 - A_S / A_0) \times 100$$

The DPPH$^{\bullet}$ stock solution was freshly prepared before each reaction to reduce the loss of free radical activity during the experiment.

ABTS assay: The ABTS method followed (Scalzo et al., 2005) and is based on the capacity of the test samples to scavenge the coloured ABTS radical cation (green ABTS$^{\bullet+}$), obtained by oxidation with potassium persulphate solution for 12-16 h at 4 °C away from light. The absorption peak of ABTS$^{\bullet+}$ is at 734nm and the addition of antioxidants reduces it to its colourless form. On the day of the assay, the ABTS•+ solution was diluted with ethanol until absorbance of 1.00 ± 0.02 at 734 nm. 25 µL of sample extracts were added to 1 mL of the ABTS$^{\bullet+}$ solution. The decrease in absorbance was measured after 7 min of incubation at 734 nm. Ethanol was used to set the zero. The radical scavenging activity of the samples tested, expressed as a percentage of the inhibition of ABTS$^{\bullet+}$ (I%), were calculated using the formula:

$$I\% = [(A_0 - A_S) / A_0] \times 100$$

For both assays, a linear regression was determined and used to calculate the IC_{50} value. Low IC_{50} values indicate greater antioxidant activity.

3. Results and Discussion

All results presented are averages (±SD) of three repetitions. The treatments were compared by performing a Two-Way factorial ANOVA (Analysis of Variance) on the phytochemical composition of the extracts and a Two-Way ANOVA on the antioxidant activities measured. This was followed by the post-hoc Tukey HSD (honest significant difference) test (95% confidence level) to compare the effect of different conditions on the parameters measured. Values of $p<0.05$ were accepted as significant. The ANOVA analyses were performed in R, Tukey HSD with the R package agricolae (De Mendiburu, 2014) and the graphs were made using the R package ggplot2 (Wickham, 2009).

3.1. Effect of the gamma irradiation and extraction technique on the phytochemical composition in thapsigargins

The results show that across all the three extraction methods, gamma irradiation had no significant effect on the phytochemical composition of the extracts obtained from *T. garganica* (Figure 2A, Table 1, Table 2). Thapsigargin is today isolated from plant grown in Ibiza (Spain) and shipped around the world for extraction. We can suggest that in the future

the plant material can be safely sterilised by gamma irradiation and thereby add to the conservation of the product during transport.

Figure 2B illustrates the effect of the extraction methods on the chemical extracts, which was found to be significantly different from each other in both root extracts (F-value=7.21, P-value=0.001) and leaves extracts (F-value=4.47, P-value=0.01) (Table 2). Simple extraction with liquid nitrogen (SE) of dried *T. garganica* roots and leaves proved to be the least effective method to extract bioactive compounds from small amounts of plant material, with no significant difference between microwave assisted extraction (MAE) and classical maceration (CM). MAE however presents the advantage of being rapid and reproducible as well as requiring less energy than conventional methods like CM (Azwanida, 2015). MAE is known to cause the thermal degradation of certain analytes, but in here, it has been shown to be a suitable method for the extraction of thapsigargins as previously suggested (Benkaci-Ali et al., 2006). The only chemical variations observed between the different extracts were that the Tg content in both the leaves and the roots was higher than Tc and Nt; dried roots of *T. garganica* have significantly higher levels of Tg than in the dried leaves ($P<0.00$, Figure 2B). Nt was the least abundant compound in all the samples. This has already been reported (Smitt et al., 1995), but new localities have been investigated in this study. Clear differences were also seen between the two study regions for the roots only, with the root extracts from the Béjaia region consistently having larger quantities of the three compounds studied (Figure 2B). Locality has previously been shown to have an effect on the phytochemical composition of *T. garganica* roots (Drew et al., 2012; Smitt et al., 1995), but the cause(s) of these variations have not yet been identified. We hypothesise that there are biological and environmental factors responsible for these fluctuations. Further investigations are needed to determine the best time to harvest *T. garganica* to optimise Tg extraction, considering that the compound remains extremely expensive at € 187 per mg (Sigma-Aldrich).

3.2. Effect of gamma irradiation on the antioxidant properties of T. garganica

Gamma irradiation was found to have no significant effect on the antioxidant activity of *T. garganica* root extracts (Table 3). However, there was significant difference between the scavenging activities of both leaf and root extracts between the Médéa and Béjaia regions, for both the $ABTS^{\bullet+}$, leaves (F-value = 4.97, P-value = 0,05), roots (F-value = 8.68, P-value = 0.01) and DPPH assays, leaves (F-value = 9.66, P-value = 0.01), roots (F-value = 59.77, P-value = 0.00). It was noted that IC_{50} values for the DPPH assay were higher than those obtained with the $ABTS^{\bullet+}$ assay for the root extracts (Table 3). It has been previously shown

that DPPH assays are a rapid and reliable test for the antioxidant capacity of plant extracts, but also an advantageous assay applicable to both hydrophilic and lipophilic environments. The leaf extracts had a much higher scavenging activity than the root extracts with those from the Béjaia region generally higher than the extracts from Médéa. This again shows that there are biological or environmental factors responsible for these fluctuations. As *T. garganica* and other Algerian medicinal plants have been proposed as potential sources of natural antioxidants (Djeridane et al., 2006). This shows that whilst gamma irradiation can be used as a sterilisation method, the locality where a plant is collected can affect its antioxidant potential.

4. Conclusion

A difference in the chemical composition of Thapsigargins was observed between different tissues of *T. garganica*. The highest amount of Thapsigargin was found to be in the roots of samples collected in Béjaia. There was a significant effect of locality on the phytochemical composition of the roots but not in the leaves. Locality also affected the antioxidant properties of both the leaf and root extracts.

Of the extraction methods used, MAE and CM were equally effective and more efficient than SE to extract bioactive compounds from small amounts of plant material. Gamma irradiation had no significant effect on the phytochemical composition of *T. garganica* as well as the antioxidant activity of the extracts.

5. Author contributions

A. Mohamed Mohamed Ibrahim and K. A. Martinez-Swatson established the major part of the results and contributed equally to the manuscript. A. Mohamed Mohamed Ibrahim conducted fieldwork for collects of samples and extraction, prepared samples for HPLC analysis, conducted the antioxidant activity test. K. A. Martinez purified the chemical compounds used as standards and ran the HPLC analysis. F. Cozzi directed and supported the HPLC analysis. F. Benkaci-Ali conceived the project and contributed to the manuscript. F. Zoulikha supervised the writing. H. T. Simonsen initiated, directed and supported the research and writing of the manuscript. All authors edited and approved the final manuscript.

6. Acknowledgments

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8. Table legends

Table 1: Comparison of the different extraction methods and gamma irradiation doses on the samples from the two study areas Médéa and Béjaia. Values are the mean of three replicates ± standard deviation (SD) and are expressed as the percentage of compound in the dry weight of the sample (DW%), $P < 0.05$. Tg = Thapsigargin, Tc = Thapsigargicin, Nt = Nortrilobolide, CM = Classical maceration, MAE = Microwave assisted extraction, SE = Simple extraction with liquid nitrogen, MR = Médéa roots, ML = Médéa leaves, BR = Béjaia roots, BL = Béjaia leaves. Gamma irradiation doses are given in KGy, significance $P < 0.05$ is compared to the control 0 KGy. Superscript letters within the same row indicate significant ($P < 0.05$) differences in the compound yields between the extraction methods.

Table 2: Factorial two-way analysis of variance on the effect of γ-irradiation (gamma irradiation), extraction method and locality on the chemical variation of the extracts from *T. garganica* with significance displayed as *** $P > 0.00$, ** $P > 0.001$, * $P > 0.01$. df = degrees of freedom, Sum sq = sum of squares, Mean sq = mean square, F_S = F statistic.

Table 3: The effect of gamma irradiation on the antioxidant activity of samples obtained by CM with methanol. Values are represented as the mean IC_{50} (mg/L) value of three replicates ± standard deviation at 5% significance level. DPPH = 2,2-diphenyl-picryl-hydrazyl assay, ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt assay. Gamma irradiation dosages (D) are given in KGy. MR = Médéa roots, ML = Médéa leaves, BR = Béjaia roots, BL = Béjaia leaves.

9. Figure legends

Figure 1: Illustration of structures of the three main sesquiterpene lactones in *Thapsia garganica* L. Thapsigargin (Tg), thapsigargicin (Tc), and nortrilobolide (Nt).

Figure 2: **A** – Stacked bar chart showing the effect of gamma irradiation on Thapsigargin (Tg), thapsigargicin (Tc) and nortrilobolide (Nt) levels in the different extracts obtained by the different extraction methods. The dose of irradiation used is shown in kGy with the control (NT = no treatment) and each dosage extraction is represented in a different colors. The amounts presented are relative to the individual samples run. For each irradiation dosage, the extraction methods are represented with shading with the lightest shading being classical maceration (CM), the middle shading is microwave assisted extraction (MAE) and the darkest shading is simple extraction (SE). The size of the bars indicates the percentage of extraction yield, each treatment is responsible for, relative to each compound.
B – Bar plots to show the effect of the extractions methods used on the extraction yield of thapsigargin (Tg), thapsigargicin (Tc), and nortrilobolide (Nt) on samples from the two locations. MR = Médéa roots, ML = Médéa leaves, BR = Béjaia roots, BL = Béjaia leaves.
In both charts, the amount of the compounds was calculated as the percentage of the compound in the dry weight of the plant material (extraction yield).